Expression of a High-Affinity Sulfate Transporter in *Brassica juncea* Affects Metal Tolerance and Accumulation

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Abstract

The *Stylosanthes hamata* SP1 gene encodes a high-affinity sulfate transporter located in the plasma membrane. In this study the *S. hamata* SP1 gene was constitutively expressed in *Brassica juncea* (Indian mustard) to investigate its importance for tolerance and accumulation of various oxyanions that may be transported by SP1 and of cadmium, which is detoxified by sulfur-rich compounds. The transgenic SP1 lines SP1-12C and SP1-4C were compared with wildtype *B. juncea* for tolerance and accumulation of arsenate, chromate, tungstate, vanadate and cadmium. As seedlings the SP plants were significantly less tolerant to Cd, Mo and V compared to wildtype plants. They accumulated significantly more Cd and W, and somewhat more Cr and V (NS). Mature SP plants were less tolerant than wildtype plants to Cd and Cr. They accumulated significantly more Cr in their shoots and somewhat more V and W (NS); shoot Cd accumulation was significantly lower than in wildtype, and As levels were somewhat reduced (NS). In roots, mature SP plants accumulated more Cd, Cr and W than wildtype plants. Compared to wildtype plants, sulfur accumulation was enhanced in roots of SP1 plants but not in shoots. Together these results suggest that SP1 mediates uptake in roots rather than root-shoot translocation, and that SP1 can facilitate uptake of other oxyanions in addition to sulfate. Since SP1 overexpression led to enhanced accumulation of Cr, Cd, V and W, this approach shows some potential for phytoremediation, especially if it could be combined with enhanced metal translocation and tolerance.

Introduction

Toxic metals and metalloids are increasingly released into the environment by human activities such as industry, mining operations, use of ammunition, traffic and agriculture, resulting in contamination that threatens natural ecosystems and human well-being (Lantzy and Mackenzie, 1979; Nriagu, 1979; Ross, 1994). A relatively new technology for environmental cleanup is phytoremediation, which uses plants and their associated microbes to extract, degrade or stabilize pollutants. Metal extraction into harvestable plant tissues may be further enhanced by genetic engineering. Already, transgenic plants with enhanced metal tolerance and accumulation have been created via (over)expression of metal transporter proteins (Samuelsen et al., 1998; Arazi et al., 1999; Van der Zaal et al., 1999; Curie et al., 2000; Hirschi et al., 2000). The purpose of this study was to test the role of the sulfate transporter in tolerance to and accumulation of various metal(loid)s.

Sulfur is an essential element for plant primary metabolism as a structural component of proteins and lipids, antioxidants, regulatory molecules, metal-binding molecules and cofactors and coenzymes. Plants take up sulfur in the form of sulfate. After uptake most sulfate is incorporated into organic molecules via the sulfate assimilation pathway. It is activated by ATP sulfurylase to form adenosine phosphosulfate, followed by a reduction by APS reductase to form free sulfite, which is coupled to O-acetylserine to form cysteine. (Setya et al., 1996). Cysteine can be incorporated into proteins, further metabolized to methionine and its derivatives, or used for the production of the antioxidant glutathione (GSH), and the metal-binding peptides phytochelatins (PCs). Under conditions of oxidative stress such as the presence of heavy metals there is an increased demand for reduced S

compounds like GSH and PCs, and genes involved in uptake and reduction of sulfate are upregulated at the transcription level under these conditions (Leustek et al., 2000; Nocito et al., 2002), as are genes involved in formation of GSH and PCs (Xiang and Oliver, 1998).

The transport of sulfate over plant membranes is mediated by sulfate transporters. There are many different sulfate transporters in plants that differ in intracellular location, expression pattern, and kinetic properties. In Arabidopsis 14 sulfate transporters have been reported that can be divided into five distinct groups with different kinetic properties (Hawkesford, 2003). Sulfate can enter plants via a group 1 high-affinity sulfate transporter in the plasma membrane (Smith et al., 1995; Shibagaki et al., 2002; Yoshimoto et al., 2002). Group 1 high-affinity sulfate transporters are expressed mainly in plant roots, and are up-regulated under sulfur starved conditions (Yoshimoto et al., 2001).

There is evidence that sulfate transporters can also mediate the transport of related oxyanions: all sulfate transporters tested can also transport selenate (Smith et al., 1995; Hawkesford, 2003), and sulfate transport is inhibited by selenate, arsenate, chromate, molybdate, and tungstate (Wilson and Bandurski, 1958; Leustek, 1996). If sulfate transporters indeed transport other oxyanions, their constitutive overexpression may lead to enhanced uptake of these elements, a property that would be desirable for phytoremediation. In addition, it is feasible that constitutive expression of sulfate transporters leads to enhanced production of S-rich metal binding peptides (GSH, PCs), which may enhance metal tolerance and accumulation.

To investigate the effect of overexpression of a sulfate transporter on metal tolerance and accumulation, *Brassica juncea* (Indian mustard) plants were engineered to constitutively express the *Stylosanthes hamata* SP1 gene (Smith et al., 1995), encoding a high-affinity

sulfate transporter that is thought to be involved in root sulfate uptake over the cell membrane. The resulting SP1 transgenic plant lines were compared to wildtype (WT) *B. juncea* with respect to tolerance and accumulation of As, Cd, Cr, Mo, V, and W, supplied individually to seedlings or mature plants.

Materials and Methods

Materials

Brassica juncea (cv. Indian mustard) wildtype seeds were from accession no. 173874, North Central Regional Plant Introduction Station (Ames, IA). Transgenic SP1 plants were obtained via transformation of plants from this same accession number with a DNA construct containing the *Stylosanthes hamata* SP1 gene (Smith et al., 1995) under the 35S CaMV constitutive promoter. The kanamycin-resistance gene (*nptII*) under control of the nopaline synthase promoter was used as a marker gene. Hypocotyls of *B. juncea* were transformed using *Agrobacterium tumefaciens*-mediated transformation as described in Pilon-Smits et al. (1999). Seven independent transgenic lines were obtained; two of them, SP1-4C and SP1-12C were selected for further studies.

The chemical forms and concentrations of the metal(loid)s used for the seedling treatments were 25 mg L^{-1} arsenic(V) as Na_2HAsO_4, 10 mg L^{-1} cadmium as $CdSO_4$, 5 mg L^{-1} chromium as K_2CrO_4, 20 mg L^{-1} vanadium as Na_3VO_4, 85 mg L^{-1} molybdenum as $(NH_4)_6Mo_7O_{24}$, and 100 mg L^{-1} tungsten as Na_2WO_4. The same chemical forms of metal(loid) were supplied to mature plants in a hydroponic system at 16 mg L^{-1} As, 5.6 mg L^{-1} Cd, 6 mg L^{-1} Cr, 6 mg L^{-1} V, 50 mg L^{-1} W. No precipitation was observed when these metal salts were added to agar medium or nutrient solution.

Northern Blotting

Expression of the *S. hamata* SP1 gene in the two SP1 plant lines was analyzed at the mRNA level essentially as described by Van Huysen et al. (2003). Total RNA from 3-week-

old wild type and transgenic (SP1-4C and SP1-12C) *B. juncea* plants was isolated using the TRIzol reagent method (Invitrogen, Carlsbad, CA). Ten μg of total RNA was separated on a 1% (w/v) agarose gel containing 4% formaldehyde, and transferred to a nylon membrane (Hybond N; Amersham). The RNA was cross-linked to the filter by exposing it to UV light in a Stratalinker (Stratagene) and probed with a ^{33}P-labelled 1.0 kb sulfate permease cDNA obtained using the SP1-F (5'CCACCTAAGCAGACACTCTTCC-3') and SP1-R (5'-CATGGAAGAAGATTTCATTAGC-3') primers. Radioactive probe was synthesized with a DECAprime II labelling kit from Ambion (Ambion Inc, Austin, TX) using nanonucleotide random primers. Hybridization was performed at 42^{0}C in a solution containing 50% formamide, 5X SSPE, 5X Denhardt's solution, 0.1% (w/v) SDS, and 100μl/ml salmon sperm DNA. After hybridization, the membrane was washed with 0.1 X SSC and 0.1% SDS at 65^{0}C, and radioactive bands were visualized and quantified in a PhosphorImager (STORM, Molecular Dynamics, Sunnyvale, CA). As a control for loading, the 28S ribosomal RNA band was quantified from the ethidium bromide-stained gel, using Gel-Pro Analyzer (Media Cybernetics, Silver Spring, MD). The intensities of the sulfate permease bands were quantified using ImageQuant (Molecular Dynamics, Sunnyvale, CA). The relative expression levels of sulfate permease were then calculated as a ratio of the detected sulfate permease bands to the amount of 28S ribosomal RNA.

Metal tolerance and accumulation experiments

Seedlings: To compare SP1 transgenic plant lines to wildtype (WT) *B. juncea* plants for metal tolerance at the seedling level, seeds of each plant line (SP1-4C, SP1-12C, and WT) were surface-sterilized as described by Pilon-Smits et al. (1999) and grown on agar medium

containing one of the selected metals. The seeds were sown in a grid pattern of six by six in Magenta tissue culture boxes (Sigma, St. Louis, USA). Each experiment was performed in parallel with its own control, i.e. using growth medium lacking the additional metal or metalloid. The agar medium contained half-strength Murashige and Skoog (MS) salts and vitamins (Murashige and Skoog, 1962), 10 g L^{-1} sucrose, and 4 g L^{-1} agargel (Sigma); individual metals were added to the final concentrations as described earlier in this section. These concentrations were aimed to give a ~50% reduction in wildtype seedling fresh weight, corresponding with a ~75% reduction in seedling root length compared to untreated controls. The magenta boxes with the different treatments and the control boxes were randomly arranged and the seedlings were allowed to grow for seven days in a growth chamber at 25°C and a 16h light/8h dark photoperiod. Individual seedlings were then harvested, washed, and root length was measured as a parameter for metal tolerance (Murphy and Taiz, 1995). To correct for any differences between experiments, metal tolerance was expressed as relative root length, calculated as root length in the presence of the metal(loid) divided by root length on control medium. The plant material was then dried and prepared for elemental analysis.

Mature Plants: To compare metal tolerance and accumulation at the mature plant level, seeds of WT and SP1 transgenics were surface-sterilized, sown on agar medium as described above (without metal(loid) treatment) and grown for four days. The seedlings were then transferred to sand, and watered with half-strength Hoagland's nutrient solution daily (Hoagland and Arnon, 1938) in a greenhouse at 25°C, 16h light/ 8h dark photoperiod until they were five weeks old. The plants were then transferred to a greenhouse nutrient film technique setup as described by Zhu et al. (1999a). After one week of adjustment to the new

medium, the fresh weights of the plants were measured and the individual metal treatments were started. One metal was supplied at a time in the form and concentration described earlier in this section; for each experiment a control treatment without the metal(loid) was run in parallel. These metal concentrations were expected to give a ~50% reduction in fresh weight in mature plants compared to untreated controls. Ten replicate plants were used per plant line (WT, SP1-4C, or SP1-12C) per treatment. The nutrient solutions were replaced every three days for a total of 14 days, after which the plants were harvested, washed, weighed, and dried for elemental analysis.

Elemental Analysis

Plant tissues were dried for 48 hours at 70°C for elemental analysis. Five dried shoot samples of 7 seedlings per treatment were weighed and acid-digested according to the method of Zarcinas (1987). The total elemental concentrations in the digests were measured using Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES, Thermo Jarrell Ash) according to the method of Fassel (1978), using appropriate standards and quality controls. Individual shoots and roots of mature plants were ground in a Wiley mill, acid-digested and analyzed by ICP-AES as described above.

Statistical Analyses

The software program JMP-IN from the SAS Institute (Cary, NC, USA) was used for statistical analysis of metal tolerance and accumulation data. Analysis of variance (ANOVA) was performed followed by pairwise post-hoc analyses to determine which means differed

significantly (α =0.05). Statistically significant differences ($P < 0.05$) are reported in the text and shown in the figures and tables.

Results

Creation of SP1 transgenics

When *Brassica juncea* plants were transformed with a DNA construct containing the *Stylosanthes hamata* SP1 gene under the 35S CaMV constitutive promoter, seven independent transgenic lines were obtained. Two of them, SP1-4C and SP1-12C were selected for further studies. The two lines chosen express the *S. hamata* SP1 gene to different levels judging from Northern blotting (Fig. 1): SP1-12C has ~2-fold higher *SP1* expression than SP1-4C. No mRNA was detected in wildtype *B. juncea* using the *S. hamata* SP1 probe, probably due to lack of sufficient homology.

Seedling metal tolerance and accumulation

The transgenic SP1 lines were first compared with wildtype *B. juncea* (WT) with respect to metal tolerance and accumulation in seedlings (Fig. 2). The metals tested were As, Cd, Cr, Mo, V, and W. These metals were chosen because most occur as oxyanions similar to sulfate. Cadmium was included because this cation is known to be bound by S ligands in plants (Pickering et al., 2000). The forms and concentrations of the metal(loid)s used are described in the Materials and Methods section.

Both of the SP1 transgenic plant lines were less tolerant than WT to cadmium, molybdate and vanadate. There were no differences in tolerance between the plant lines when grown on media containing chromate or tungstate. The two SP1 plant lines gave inconsistent results for As, one line being more tolerant, and one being less tolerant than WT seedlings.

The SP1 transgenic lines both contained higher W levels in their shoots than WT seedlings (Fig. 3). The SP1 lines also contained higher shoot levels of Cr and V, but these differences were not significant. SP1-4C, the least arsenate-tolerant plant line, accumulated more As than SP1-12C or WT seedlings. The SP1-12C plant line accumulated more Cd than SP1-4C or WT seedlings. There were no differences in accumulation for Mo.

Supplying the seedlings with toxic levels of a metal(loid) affected the plant's ability to accumulate essential elements. Interactions between supplied metal(loid) and essential metals are shown for seedlings in Table 1, as are differences between control and treated plants for each plant line. The SP1 seedlings contained lower Cu levels than WT plants when grown on molybdate. The Fe levels were higher in SP1 seedlings than in WT when grown on Cd, vanadate or tungstate. SP1 seedlings accumulated more Mg than WT after As or Cd treatment, but less Mg than WT after V or W treatment. Sulfur concentration was higher in SP1 seedlings than in WT under Cd or Cr treatment, but lower than WT under V or W treatment. Zn levels were higher in SP1 plants than in WT when treated with As, Cd, W, and to a lesser extent (NS) Cr. Only after Mo treatment did SP1 seedlings have lower Zn levels than WT.

There were also differences between SP1 and WT plants with respect to the effect of the metal treatments on plant nutrient levels. Cu levels in WT seedlings treated with Cd or Mo were higher than their controls, whereas these treatments did not significantly affect Cu accumulation in the SP1 seedlings. The tungstate treatment reduced Fe accumulation in WT plants, while it enhanced Fe accumulation in SP1 plants. Magnesium accumulation was reduced in WT seedlings by the Cd treatment, while in SP1 seedlings it was not. Sulfur accumulation was enhanced by the Cd treatment in SP1 seedlings, but not significantly in

WT seedlings. SP1 seedlings treated with arsenate had higher Zn levels in their shoots compared to their own controls, whereas WT seedlings had reduced shoot Zn levels. SP1 seedlings treated with molybdate had reduced Zn accumulation compared to control SP1 seedlings, while the Mo treatment did not significantly affect Zn accumulation in WT seedlings.

Mature plant accumulation and tolerance - shoots

To test the role of the SP1 sulfate transporter in metal(loid) tolerance at the mature plant level, the two SP1 transgenic plant lines were compared to wildtype *B. juncea* in a hydroponic setup containing the metal(loid) of interest (Fig. 4). The form and concentration of the metal(loid) used is described in the Materials and Methods section. Molybdate was not included in the mature plant study since there were no differences in Mo accumulation at the seedling level. Metal tolerance was calculated for each plant line as plant growth in the presence of the metal as a fraction of its growth under control conditions. The SP1-12C plants were less tolerant than WT when supplied with cadmium or chromate; the SP1-4C line was intermediate in this respect. There were no differences in tolerance between the plant lines when grown on media containing arsenate, vanadate or tungstate.

There were differences in shoot accumulation of Cd and Cr between the SP1-4C, SP1-12C and WT plant lines (Fig. 5). The SP1-12C plants contained less Cd in their shoots when treated with Cd, and more Cr when treated with Cr compared to WT plants. No differences were found between the plant lines with respect to shoot As, V or W accumulation, although the V and W levels in the shoots of SP1 plants were somewhat higher, and As levels lower compared to WT plants.

Table 2a shows interactions in mature plant shoots between supplied metal(loid) and essential elements. SP1 plants grown in solution with Cd or W had less Fe in their shoots than WT plants. Shoots of SP1-12C plants contained less S than WT plants after treatment with Cd or vanadate; SP1-4C plants were intermediate in this respect. While the Cd treatment significantly reduced Mg and S levels in the shoots of the SP1 plants compared to control conditions, it did not in WT plants. Also, vanadate significantly reduced shoot Zn levels in SP1 plants but not in WT plants.

Mature plant metal accumulation - roots

The roots of the SP1 plants contained higher Cr levels than WT roots (Fig. 6). SP1-4C plants also accumulated Cd and W to higher concentrations than WT plants, and the SP1-12C plants were intermediate in this respect. As and V root accumulation was not significantly different between the plant lines.

Root accumulation of the essential elements are shown in Table 2b. There were higher levels of Mg in the roots of SP1 plants compared to WT roots after all of the treatments. Also, SP1 plants contained more S in their roots than WT plants after treatment with As, Cd, Cr or V, and more Cu than WT roots when treated with Cd. Zinc levels were greater in SP1-4C roots than in WT roots when the plants were grown in solution with Cd or Cr; SP-12C plants were intermediate on both treatments. Root Fe levels in SP1 plants were lower than in WT plants when treated with arsenate, chromate or vanadate. Vanadate-treated SP1-12C plant roots also had lower root Zn levels than WT; SP1-4C plants were intermediate. Arsenic treatment enhanced root S and Mg levels in the SP1 plant lines compared to control conditions, but in the WT plants it did not.

The root-shoot translocation factors for the supplied metal(loid)s are shown in Table 3a. Of the metals tested W was translocated from the roots to the shoots to a much larger extent than the other elements: S/R ratios were around 1 for W. Arsenic was translocated roughly ten-fold less, Cr and V 20-40 fold less, and Cd was translocated the least. The SP1 plants translocated Cd to a lesser degree than the WT plants. In contrast, the SP1-12C plants translocated more Cr and more V than the other plant lines. Sulfur translocation (Table 3b) was lower in SP1 lines treated with As, Cd and V compared to the WT plants. Under control conditions the SP1 plants also tended to have a lower degree of S translocation compared to WT plants.

Discussion

The *S. hamata* SP1 transporter is a group 1 high-affinity sulfate transporter expressed in the cell membrane, primarily in root cells (Smith et al., 1995). In this study *SP1* was constitutively expressed in order to gain insight into the involvement and importance of this transporter for uptake and translocation of sulfate, other supplied oxyanions, and Cd. Results from experiments with seedlings suggest that the SP1 transporter can mediate the uptake of WO_4^{2-} and possibly of VO_4^{3-} and CrO_4^{2-} since both SP1 lines accumulated more W and to a lesser extent more Cr and V compared to the WT seedlings. Treatment with arsenate, chromate and vanadate, as well as Cd, generally enhanced seedling S levels. This may have been due to upregulation of endogenous sulfate transporters. Such upregulation is triggered by S deficiency, particularly by low levels of reduced S compounds such as GSH (Lappartient et al., 1999). Lower levels of reduced S compounds after metal treatment could be explained either by competition of the supplied oxyanions with S analogs for S-metabolism enzymes, or by enhanced use of GSH for detoxification of the elements. It is known that under metal stress GSH is used to form phytochelatins, which bind As and Cd and perhaps other metals (Pickering et al., 2000). Cadmium treatment is known to lead to induced expression of sulfate transporters via its interactions with reduced S compounds. The higher Cd levels observed in the SP1 seedlings compared to WT suggest that their higher S levels facilitated Cd accumulation. The higher S levels did not appear to help SP1 seedlings detoxify the metal, since Cd tolerance was not enhanced but rather decreased in SP1 plants, probably due to the direct toxic effects of their higher Cd levels. Tolerance to molybdate and vanadate was also lower in SP1 seedlings than in WT. For V this may be due to their

somewhat higher shoot V levels. The lower Mo tolerance can not be readily explained since shoot Mo levels were comparable. It is possible that seedling root Mo levels were higher; this was not analyzed for lack of sufficient plant material.

From results with mature plants it appears that the SP1 transporter facilitates uptake of the oxyanions WO_4^{2-}, CrO_4^{2-}, and perhaps VO_4^{3-}. Constitutive expression of SP1 also facilitated uptake of SO_4^{2-} and Cd into roots, but not their translocation to the shoot. Thus, the results from seedlings and mature plants were fairly consistent, except that the additional Cd and S taken up by SP1 roots was not translocated to the shoot in mature plants. The lower translocation of Cd and S (as well as As) in SP plants may be due to enhanced Cd (and As) sequestration in the roots by the S-rich thiols GSH and PCs, preventing further transport in/into the vascular tissue. It is also possible that the endodermis, which acts as a barrier for root-to-shoot transport, is still developing in 7d-old seedlings. Once the endodermis is fully established the root-to-shoot translocation depends on export of the ions out of the root symplast into the root xylem. This process is likely not mediated by SP1 and thus limited by the activity of endogenous transporters.

Translocation of sulfate to the shoot via the xylem is thought to be facilitated by sulfate transporters from groups 4, 3 and 2 in Arabidopsis roots, involved in vacuolar efflux (sultr4;1 and 4;2) and xylem loading (sultr3;5 and 2;1) respectively (Takahashi et al., 1997, 2000, pers. comm.). Sulfate uptake from the shoot xylem into leaf mesophyll cells may involve the combined action of group 2 and 3 sulfate transporters (Takahashi et al., 1999; Grossman and Takahashi, 2001). These same sulfur transporters may be involved in translocation of the different oxyanions used in this study. The affinity of the individual transporters for the different oxyanions likely varies. This would explain both the differences in translocation

between seedlings and mature plants and between the different elements. Tungsten differed remarkably in translocation from the other elements tested, showing a root-shoot ratio that was close to 1. Perhaps there is no barrier limiting root-to-shoot translocation for this element, e.g. if it crosses membranes via passive transport.

The lower tolerance of the SP1 plants to Cd may in part be due to Cd-induced deficiency of micronutrients. Cd treatment of mature plants resulted in a decrease in shoot concentration of Cu and Mg in SP1 plants but not in WT, and a decrease in Fe and Zn in all plant types but more so in SP1 plants. The lower metal micronutrient levels in SP shoots are expected to impair photosynthesis and thus productivity. In addition, the higher root Cd levels in SP1 plants may have led to a direct toxic effect, e.g. by mediating oxidative stress. The lower tolerance of the SP1 plants to Cr is not apparent from nutrient levels and may be due to a direct toxic effect of chromate at the cellular level, since Cr levels were higher in SP1 plants.

Does constitutive expression of a high-affinity sulfate transporter like SP1 show any promise for breeding plants with enhanced phytoremediation capacity? The SP1 plants showed enhanced uptake of Cr, Cd, V and W, which would be an attractive property for use in phytoextraction, where plants are used to accumulate pollutants followed by harvesting of the plant material. The reduced tolerance of the SP1 plants to Cd and Cr and their inability to translocate the accumulated Cd to the shoot may be overcome by concomitant overexpression of genes that enhance metal tolerance or translocation. Enhanced tolerance to Cd, for instance, may be conferred by overproduction of phytochelatins (Zhu et al., 1999a,b). Simultaneous expression of two genes, one conferring enhanced uptake and one enhanced tolerance has been used successfully for As (Dhankher et al., 2002). In addition, if nutrient

deficiency appears to be a cause of the reduced tolerance, this effect may be alleviated by fertilization with the nutrients in question. Increased translocation may be achieved by simultaneous overexpression of a transporter involved in xylem loading or unloading. Still much remains to be discovered about the nature of these transporters, but as mentioned above, certain group 2, 3 and 4 sulfur transporters may be possible candidates (Grossman and Takahashi, 2001).

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Figure legends

Figure 1. Transcript levels of *S. hamata SP1* in transgenic *B. juncea* lines. Total RNA was isolated from 3 week-old wildtype (WT) and sulfate permease overexpressing (SP1-4C and SP1-12C) *B. juncea* plants. A: Ethidium bromide-stained gel showing total RNA loading; B: Autoradiogram of RNA blot after hybridization with ^{33}P-labelled *SP1* cDNA; C: Relative expression of the *S. hamata* SP1 gene in *B. juncea* transgenics. The intensity of the bands shown in A and B were scanned and the ratio of B/A calculated.

Figure 2. Seedling metal tolerance for wildtype (WT) and sulfate permease (SP1-4C and SP1-12C) overexpressing *B. juncea* plants. Shown is the ratio of seedling root length grown on medium containing metal(loid) relative to root length on control medium. The form and concentration used for each metal(loid) is described in Materials and Methods. Shown are means $\pm$ SE (n=36). The letters above the bars indicate statistically significant differences between groups (ANOVA with pairwise post-hoc analyses, $\alpha = 0.05$).

Figure 3. Seedling metal accumulation for wildtype (WT) and sulfate permease (SP1-4C and SP1-12C) overexpressing *B. juncea* plants. Shown is the shoot metal concentration after 7d of growth on agar medium supplied with a metal(loid) to a concentration as indicated in Materials and Methods. Shown are means $\pm$ SE (n=5 samples pooled from 14 plants each). The letters above the bars indicate statistically significant differences between groups (ANOVA with pairwise post-hoc analyses, $\alpha = 0.05$).

Figure 4. Mature plant metal tolerance for wildtype (WT) and sulfate permease (SP1-4C and SP1-12C) overexpressing *B. juncea* plants. Shown is the ratio of plant fresh weight when grown on medium containing metal(loid) relative to its fresh weight on control medium. The form and concentration used for each metal(loid) is described in Materials and Methods. Shown are means $\pm$ SE (n=10). The letters above the bars indicate statistically significant differences between groups (ANOVA with pairwise post-hoc analyses, $\alpha = 0.05$).

Figure 5. Mature plant shoot metal accumulation for wildtype (WT) and sulfate permease (SP1-4C and SP1-12C) overexpressing *B. juncea* plants. Shown is the shoot metal concentration after hydroponically grown plants were treated for 14d with a metal(loid) concentration as indicated in Materials and Methods. Shown are means $\pm$ SE (n=10). The letters above the bars indicate statistically significant differences between groups (ANOVA with pairwise post-hoc analyses, $\alpha = 0.05$).

Figure 6. Mature plant root metal accumulation for wildtype (WT) and sulfate permease (SP1-4C and SP1-12C) overexpressing *B. juncea* plants. Shown is the root metal concentration after hydroponically grown plants were treated for 14d with a metal(loid) concentration as indicated in Materials and Methods. Shown are means $\pm$ SE (n=5 samples pooled from two plants each). The letters above the bars indicate statistically significant differences between groups (ANOVA with pairwise post-hoc analyses, $\alpha = 0.05$).

Table captions

Table 1. Shoot concentrations of the essential elements Cu, Fe, Mg, S and Zn (mg kg^{-1} dry weight) in wildtype (WT) and sulfate permease overexpressing (SP1-4C and SP1-12C) *B. juncea* seedlings after treatment with various metal(loid)s. Values shown represent the average and standard error of five replicate samples, collected from eight seedlings each. Superscript lettering indicates statistical differences between the plant types ($\alpha = 0.05$).

Table 2. Shoot (A) and root (B) concentrations of the essential elements Cu, Fe, Mg, S and Zn (mg kg^{-1} dry weight) in mature wildtype (WT) and sulfate permease overexpressing (SP1-4C and SP1-12C) *B. juncea* plants after treatment with various metal(loid)s. Values shown represent the average and standard error of ten replicate plants, each sampled once. Superscript lettering indicates statistical differences between the plant types ($\alpha = 0.05$).

Table 3. Root-Shoot translocation in wildtype (WT) and sulfate permease overexpressing (SP1-4C and SP1-12C) *B. juncea* plants treated with different elements, expressed as the ratio of shoot (Fig. 5) and root (Fig. 6) concentration. a) S/R ratio of the supplied metal (As, Cd, Cr, V, W); b) S/R ratio of sulfur for the various treatments.

a.

	As	Cd	Cr	V	W
WT	0.18±0.07 **a**	0.0014±0.0001 **a**	0.041±0.006 **ab**	0.013±0.003 **a**	1.02±0.405 **a**
SP1-4C	0.07±0.01 **a**	0.0007±0.00002 **b**	0.026±0.003 **a**	0.013±0.002 **a**	1.19±0.333 **a**
SP1-12C	0.08±0.02 **a**	0.0007±0.0001 **b**	0.053±0.012 **b**	0.027±0.001 **b**	1.09±0.107 **a**

b.

	As	Cd	Cr	V	W	control 1	control 2
WT	1.68±0.20 **a**	1.03±0.14 **a**	0.96±0.07 **a**	1.68±0.15 **a**	0.84±0.03 **a**	1.26±0.11 **a**	1.54±0.17 **a**
SP1-4C	1.11±0.02 **b**	0.78±0.09 **ab**	0.62±0.08 **b**	1.14±0.12 **b**	0.97±0.10 **a**	1.16±0.06 **a**	1.40±0.19 **ab**
SP1-12C	1.17±0.12 **b**	0.54±0.06 **b**	1.15±0.15 **a**	1.21±0.09 **b**	0.91±0.16 **a**	1.19±0.11 **a**	1.08±0.08 **b**

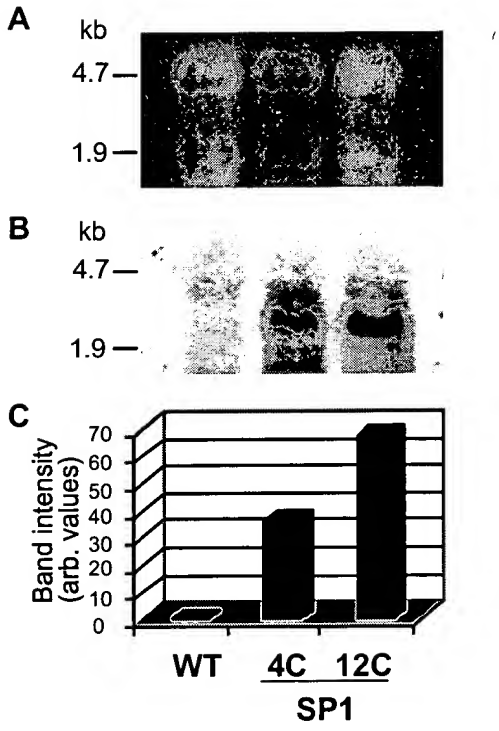

Fig. 1

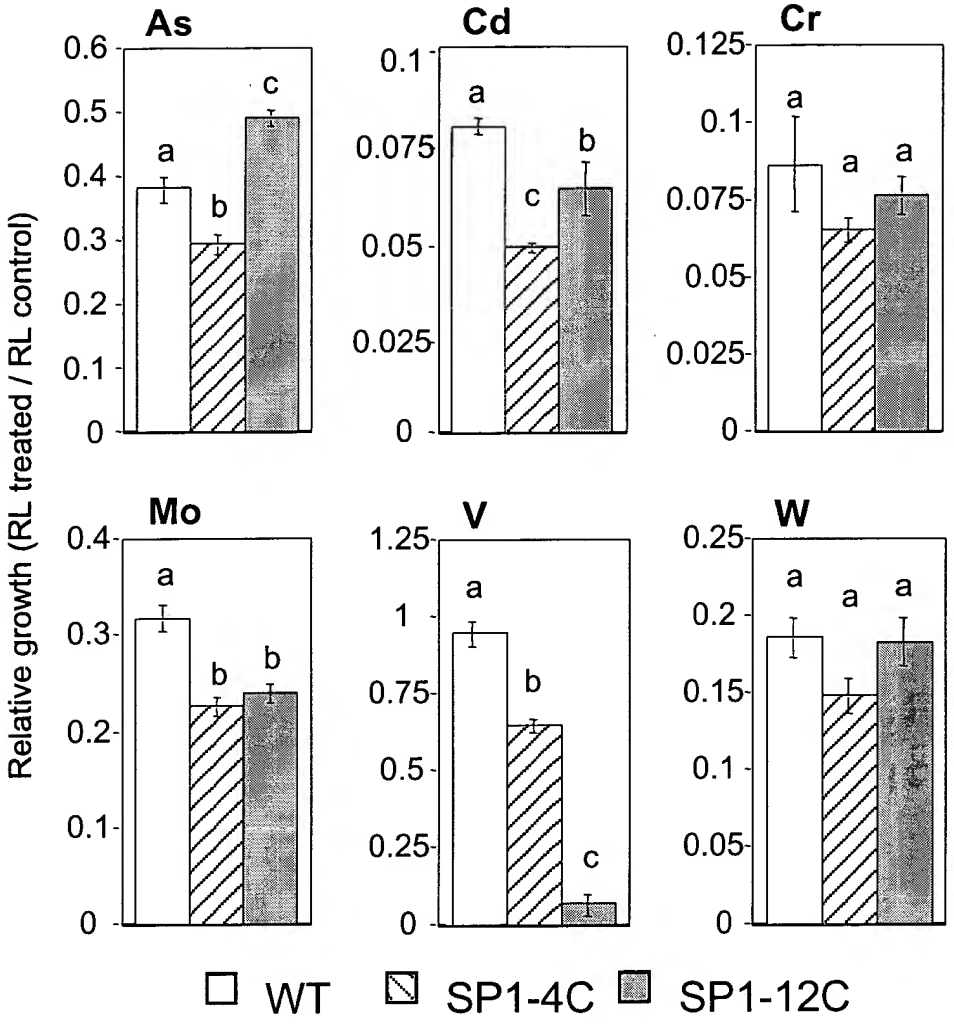

Fig. 2

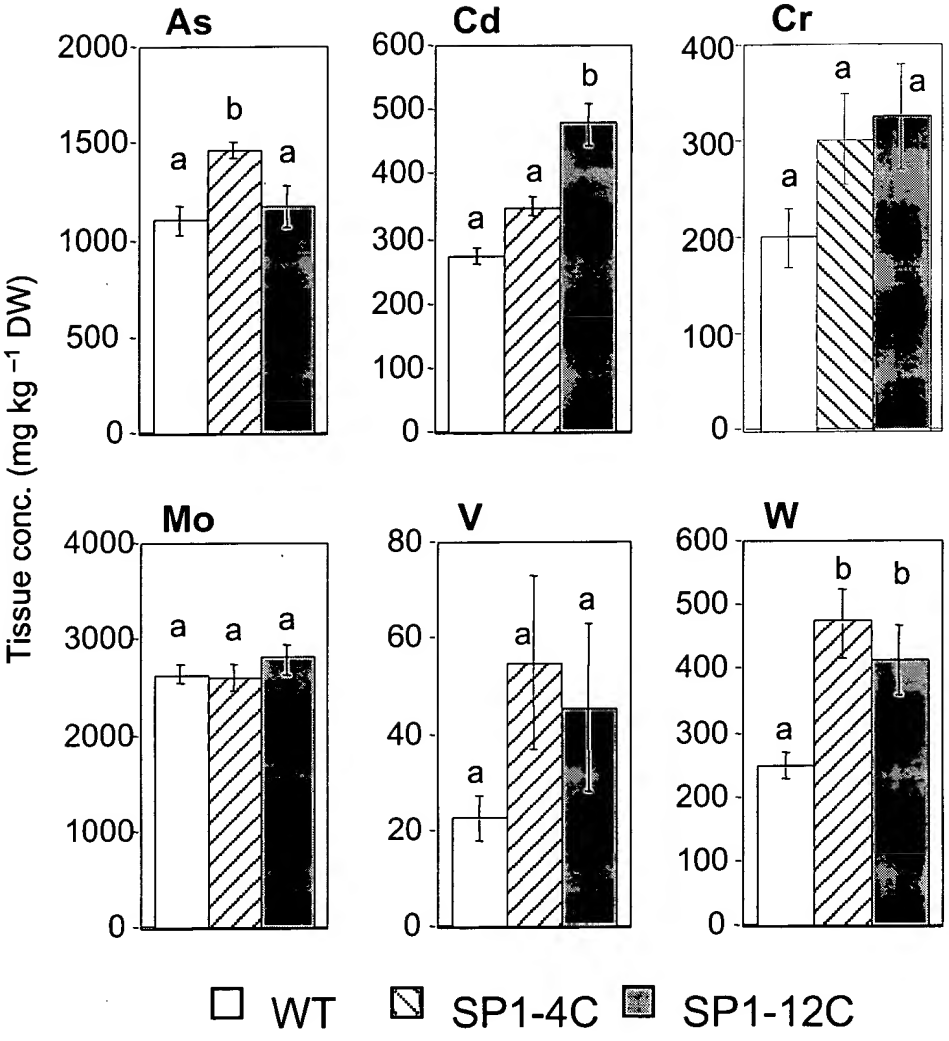

Fig. 3

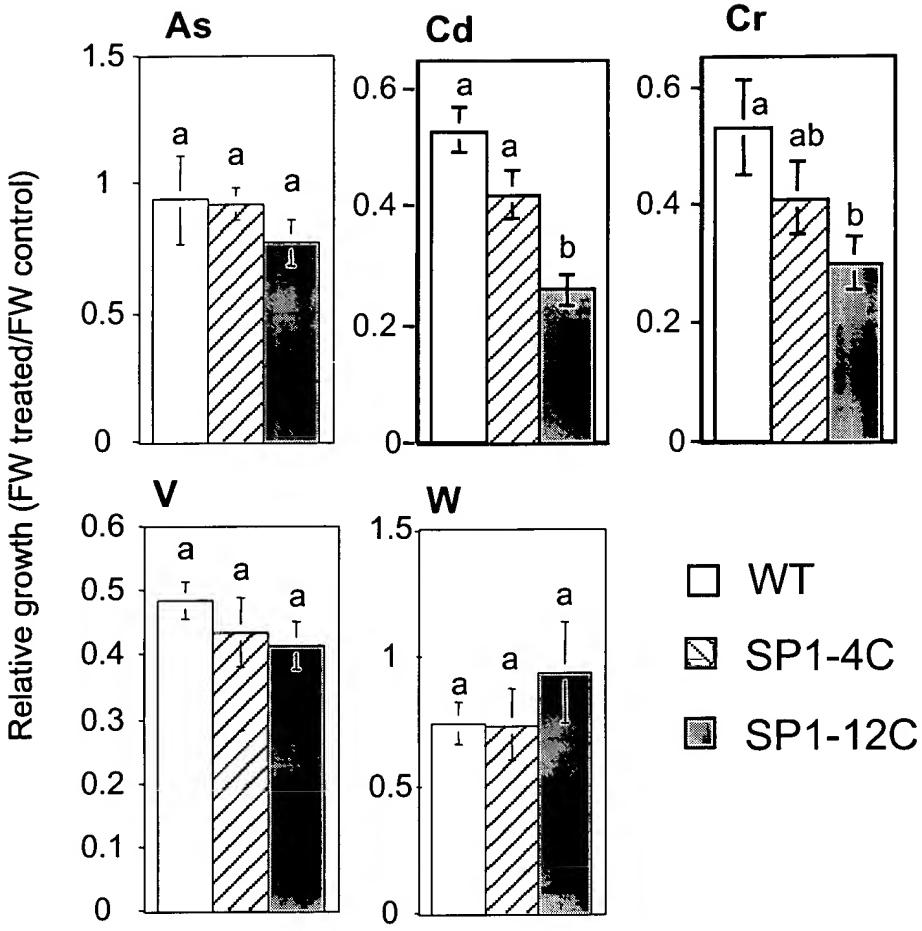

Fig. 4

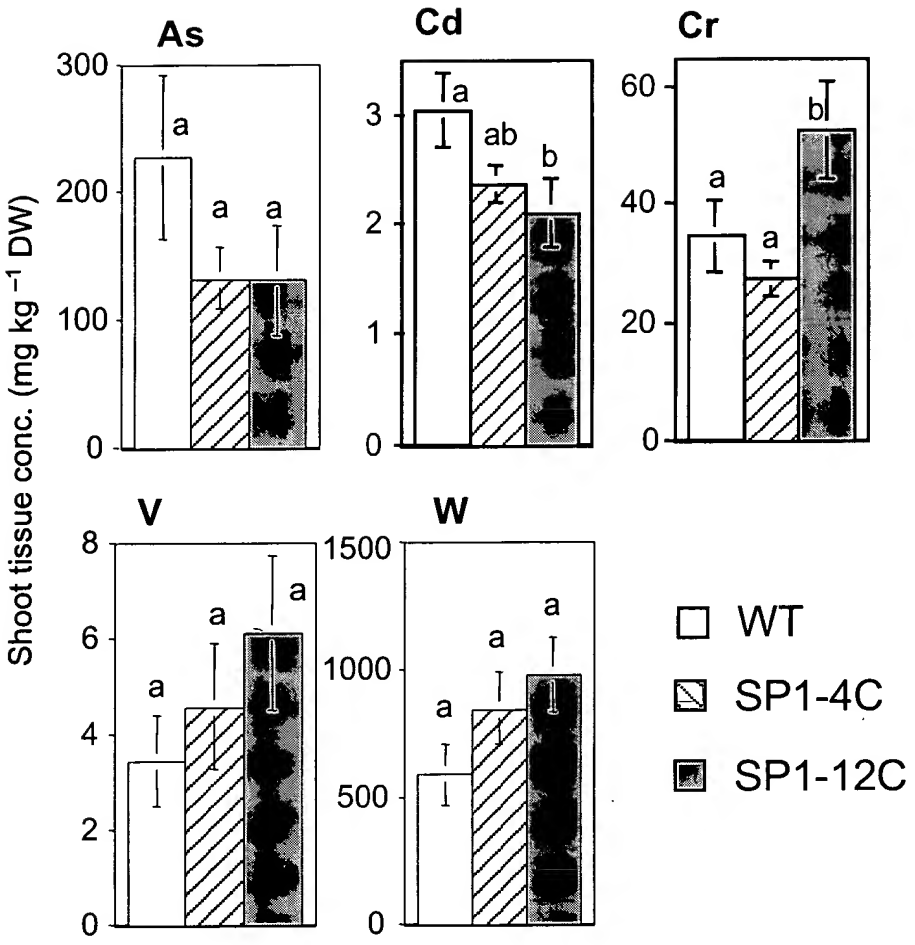

Fig. 5

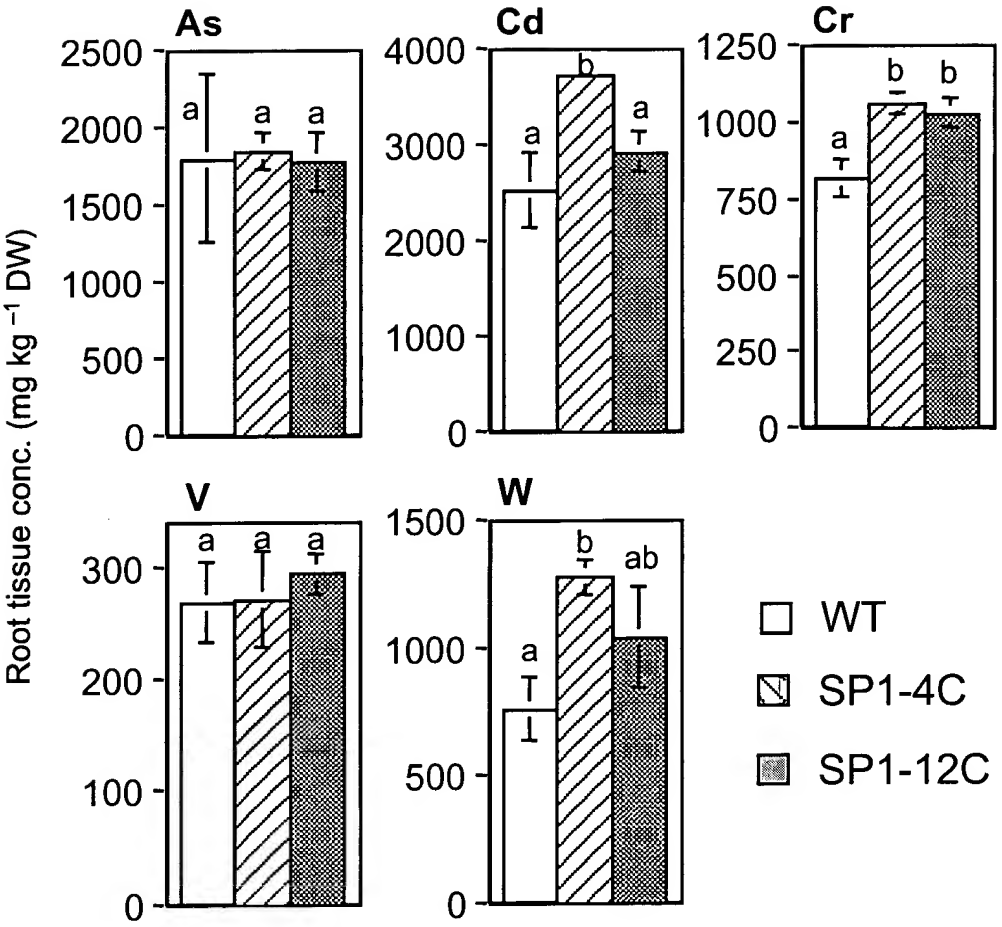

Fig. 6